[CANCER RESEARCH 56, 663–668, February 15, 1996]

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Advances in Brief

Endothelin-1 Production and Decreased Endothelin B Receptor Expression in Advanced Prostate Cancer[1]

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Abstract

The potent vasoconstrictor endothelin-1 (ET-1) is at its highest concentration in the normal human ejaculate and is associated with the progression of metastatic prostate cancer. ET-1 protein expression is detected *in situ* in 14 of 14 primary cancers and 14 of 16 metastatic sites of human prostatic carcinoma. Exogenous ET-1 induces prostate cancer proliferation directly and enhances the mitogenic effects of insulin-like growth factor I, insulin-like growth factor II, platelet-derived growth factor, basic fibroblast growth factor, and epidermal growth factor in serum-free conditions *in vitro*. The ET_A-selective receptor antagonist A-127722 inhibited ET-1-stimulated growth, but the ET_B-selective receptor antagonist BQ-788 does not. ET-3, an ET_B-selective agonist, also had no effect on prostate cancer growth. No specific ET_B-binding sites could be demonstrated in any established human prostate cancer cell line tested, and ET_B mRNA, detected by reverse transcription PCR, was reduced. The predominance of ET_B binding on human benign prostatic epithelial tissue is not present in metastatic prostate cancer by autoradiography. In human prostate cancer progression to metastases, ET-1 and ET_A expression are retained, whereas ET_B receptor expression is reduced.

Introduction

The lethal phenotype of androgen-independent human prostate cancer will claim an estimated 40,000 lives in the United States in 1995 (1). In an attempt to understand the cytokines involved in the lethal prostate cancer phenotype better, we identified ET-1[3] as an important factor in the pathophysiology of prostate cancer tumor biology previously (2). For example, plasma-immunoreactive ET concentrations are elevated abnormally in 58% of men with metastatic tumor burdens; every human prostate cancer cell line tested produces ET-1 at the levels of mRNA and protein, and exogenous ET-1 is a prostate cancer mitogen *in vitro* and enhances new bone formation *in vivo*. The ET family consists of three isoforms and four homologous cardiotoxic peptides (sarafotoxins) isolated from the venom of *Atractaspis engaddenis*; all have 21 amino acids and complete identity at 10 positions (reviewed in Ref. 3). As a growth regulatory peptide, ET-1 can influence cell proliferation directly and has potent synergy with many of the same peptide growth factors implicated in advanced prostate cancer progression (reviewed in Refs. 4–6).

This study tested the hypotheses that ET-1 expression in the androgen-refractory and lethal prostate cancer phenotype acts to enhance the effects of other autocrine peptide growth factors in prostate cancer proliferation through specific ET receptors, which can be targeted for therapy. Therefore, we tested whether ET-1 is produced in many primary and metastatic human prostate cancer tumors, evaluated the receptor pathways mediating the mitogenic and synergistic effects of ET-1 on prostate cancer proliferation *in vitro*, and quantified specific ^{125}I-labeled ET-1 binding sites in benign, malignant, and metastatic human prostate tissues.

Materials and Methods

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue sections (6-μm thick) were obtained from radical prostatectomy specimens (10 primary lesions), 16 prostate cancer metastases (7 bone, 2 liver, 2 adrenal, 2 lymph node, 1 lung, 1 splenic, and 1 subdural), and 4 primary prostate tumors from the autopsies of five men who died of prostate cancer. These sections were stained with a monoclonal (mouse) anti-ET-1 antibody (IgG_1; clone TR.ET.48.5; Affinity Bioreagents, Neshanic Station, NJ) using an immunoperoxidase method (Vectastain avidin-biotin complex kit; Vector Laboratories, Inc., Burlingame, CA). To assure specificity, the primary antibody was: (*a*) preincubated with 4 nmol ET-1 (Sigma Chemical Co., ST. Louis, MO) for immunoabsorption (negative control in all cases), (*b*) omitted, or (*c*) substituted with nonspecific mouse IgG. Immunostaining intensity was graded by two blinded observers.

Mitogenic Assays. Cell cultures and mitogenic assays were performed as described previously (2) with the following minor modifications. Five $\times 10^3$–2×10^4 human prostate cancer cells were allowed to adhere in 96-well plates, washed, and exposed to RPMI 1640 media alone (negative control), RPMI 1640 supplemented with exogenous ET-1 or ET-3 (Sigma) or the ET_A-selective [A-127722 (K_i: ET_A, 69 pM; ET_B, 114 nM); Ref. 7] and ET_B-selective [BQ-788 (50% inhibitory concentration: ET_A, 1000 nM; ET_B, 1 nM); Ref. 8] antagonists (10 pM to 100 nM). To determine whether ET-1 (10 nM) could enhance the effects of various growth factors on prostate cancer proliferation, the following human growth factors (10 ng) were screened in serum-free conditions in five human prostate cancer cell lines: EGF, basic fibroblast growth factor, IGF-I, IGF-II, and PDGF (R&D Systems, Inc., Minneapolis, MN). All conditions were run in series of four to six wells with a minimum of three experiments per cell line.

Cell Death Analysis. To determine whether ET-1 could inhibit apoptosis, quantification of DNA fragmentation indicative of apoptosis was performed in the prostate cancer cell lines following exposure to ET-1. Briefly, prostate cancer cell lines were exposed to ET-1 (1–100 nM) in RPMI 1640 or to RPMI 1640 alone for 2, 4, or 6 days. All cells were harvested (adherent and floating); half of the sample was subjected to centrifugation, precipitation, and quantification of high- and low-molecular-weight DNA as described previously (9, 10), and the rest was subjected to flow cytometric analysis (FACStar Plus; Becton Dickinson, Mountain View, CA) of apoptotic and nonapoptotic nuclei using the ApopTag *in situ* apoptosis detection kit (Oncor, Gaithersburg, MD). Dexamethasone (1 μM)-treated human peripheral blood lymphocytes, prostate cancer cell lines treated with phenylbutyrate (a known inducer of human prostate cancer apoptosis; Ref. 10), and terminal deoxynucleotidyltransferase enzyme exclusion acted as the positive and negative controls, respectively.

Received 11/1/95; accepted 1/2/96.

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[1] This work was supported by grants from the American Foundation for Urological Disease, Inc. (to J. B. N., the Dornier Scholar, and K. C-T.) and the Southern Medical Association (to K. C-T.) and by NIH Prostate Cancer SPORE Grant CA-58236.

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[3] The abbreviations used are: ET, endothelin; ET-1, endothelin-1; ET_A, ET A receptor; ET_B, ET B receptor; EGF, epidermal growth factor; IGF, insulin-like growth factor; PDGF, platelet derived growth factor; K_d, dissociation constant; B_{max}, maximal binding capacity; RT, reverse transcription; NO, nitric oxide.

^{125}I-labeled ET-1-binding Studies. Binding sites for ET-1 were evaluated using saturation and competitive binding techniques described previously for prostatic tissues (11) and cultured cells *in situ* (12, 13). Briefly, saturation studies were performed in triplicate at various concentrations (0.0625–2.0 nM) of ^{125}I-labeled ET-1 (2000 Ci/mmol; Amersham, Arlington Heights, IL). Nonspecific binding was determined in parallel assays (three times) in the presence of a final concentration of 1 μM nonradioactive ET-1. The apparent K_d and B_{max} under these *in situ* conditions were calculated by Scatchard analysis of binding data. Competition binding studies were performed in the presence of 0.1 nM (final concentration) ^{125}I-labeled ET-1, varying concentrations of unlabeled ET-1, ET_A-selective (BQ-123 and A-127722), or ET_B selective (sarafotoxin S6C and BQ-788) ligands (5×10^{-13}–5×10^{-5}) in HBSS containing 0.1% BSA. In both studies, conditions were added to confluent, washed monolayers of prostate cancer cells cultured in 24-well plates (Falcon, Oxnard, CA) or to 1×10^6 cells in 1.5-ml Eppendorf tubes for poorly adherent prostate cancer cell lines. After 60 min of incubation at 25°C, cells were extensively washed and solubilized, and cell-bound radioactivity was determined on a gamma counter (Wallac Wizard 1470, Turku, Finland) with 78% efficiency.

RT-PCR. Total RNA was isolated from prostate cancer cell lines, an immortalized human neonatal prostatic epithelial line (267-B-1), a benign prostatic hyperplasia epithelial line (BPH-1), and human placenta by the guanidium thiocyanate-phenol-chloroform extraction method. The RT-PCR was performed using a GeneAmp RNA PCR kit (Perkin Elmer/Cetus, Norwalk, CT) according to the manufacturers' instructions. Briefly, 1 μg total RNA was reversed transcribed using the random hexamer primers. The cDNA was then amplified for 25 or 35 cycles using an intron-spanning, specific ET_A or ET_B set of primers. Each cycle consisted of 95°C for 1 min (denature), 60°C for 1 min (anneal), and 72°C for 2 min (extend). The primer sets (ET_A, 5′-GATCACAATGACTTTGGCGTATTTC-3′ and 5′-CTCAAGCTGCCATTCCTTCTGTTC-3′; ET_B, 5′-CTAAAGGAGACAGGACGGCAGGATC-3′ and 5′-GATTCGCAGATAACTTCCTTTGTAG-3′) were based on published sequences of human ET_A and ET_B (14, 15). The RT-PCR samples were evaluated by agarose gel electrophoresis and Southern blot analysis, and the PCR products were cloned and sequenced to confirm product specificity.

Autoradiographic Localization of ^{125}I-labeled ET-1-binding Sites. Autoradiography was performed as described previously (16). Briefly, five human prostate tissues containing benign and malignant epitheliums were obtained from radical prostatectomy specimens, and multiple prostate cancer liver metastases obtained at autopsy were frozen immediately and stored at −80°C. Six consecutive sections (20 μm) were incubated in one of the following solutions: total ET-1 binding [0.1 nM ^{125}I-labeled ET-1 (label)], nonspecific binding (label + 1 μM ET-1), total ET_B binding (label + 1 μM BQ-123 or A-127722), and total ET_A binding (label + 0.1 μM sarafotoxin S6C or 1 μM BQ-788). Sections were exposed to Hyperfilm-^{3}H with 20-μm-thick autoradiographic ^{125}I Micro-Scales standards (Amersham). The autoradiographs were analyzed quantitatively by converting the median pixel density (Photoshop 3.0; Adobe Systems, Inc., Mountain View, CA) of sequential sections at the same histological location to radioactivity (nCi/mg), using a curve generated from the median pixel density of the calibrated standards. Specific radioactive densities were determined by subtracting nonspecific from total radioactive densities.

Results and Discussion

Immunohistochemistry. This study found that specific ET-1 immunoreactivity was present in human prostate cancer *in vivo*, independent of the clinical stage or hormonal milieu, as demonstrated by immunohistochemical staining of multiple prostate cancer lesions (Fig. 1). Specific cytoplasmic ET-1 immunostaining was present in every primary prostate cancer specimen (14 of 14) studied and in 14 of 16 lesions obtained at autopsies from 5 men who died of widely metastatic prostate cancer. This conclusion is supported by negative immunoreactivity following immunoabsorption, omission of the primary antibody, or substitution of nonspecific mouse IgG. Endothelial

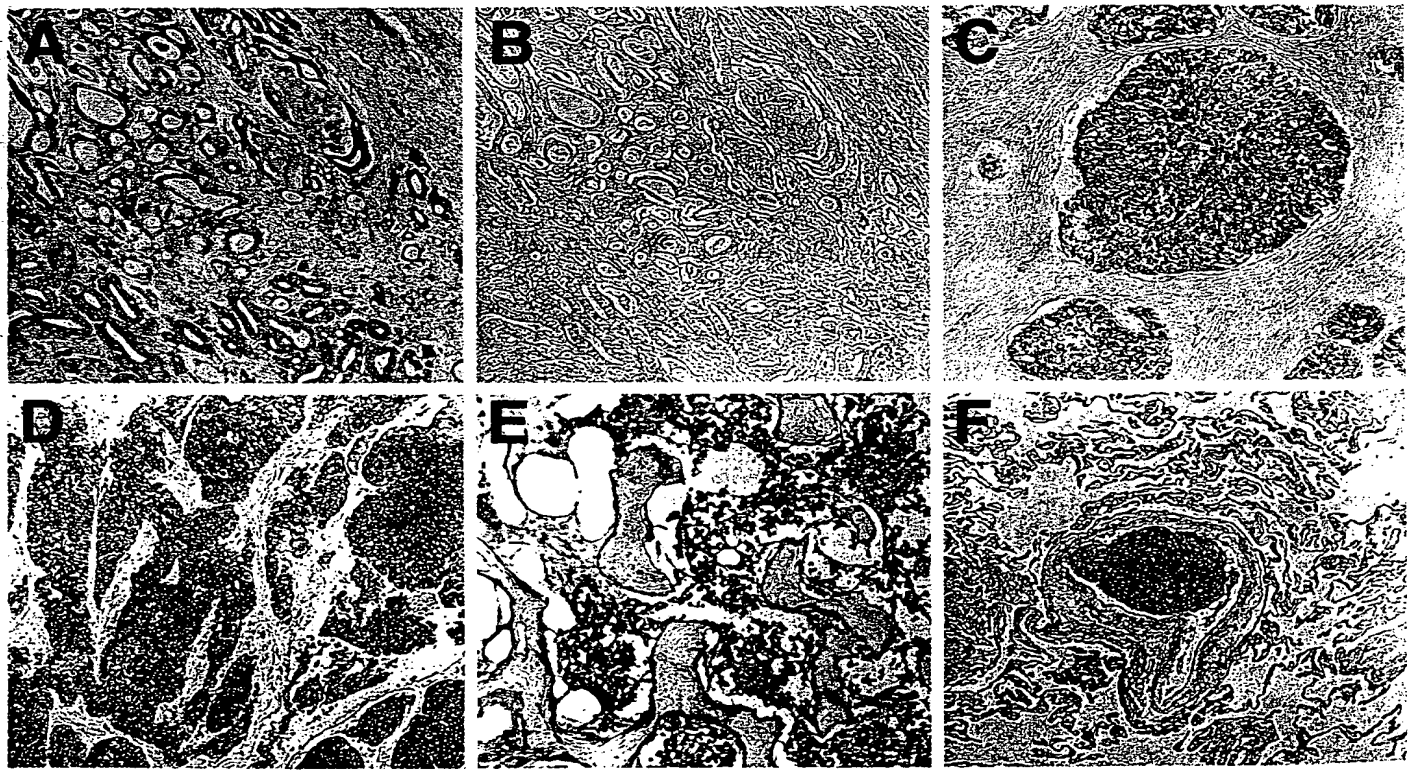
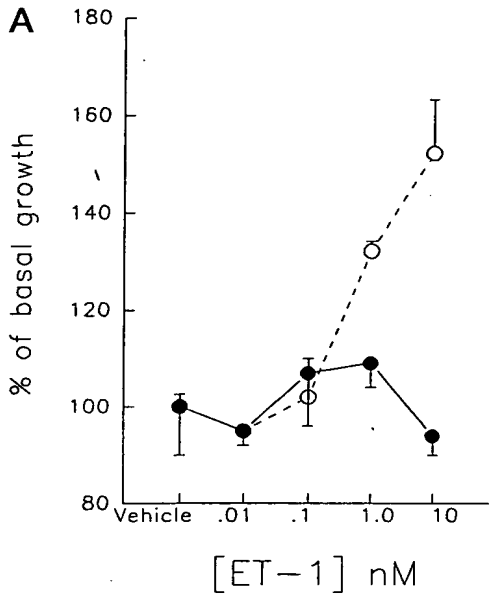
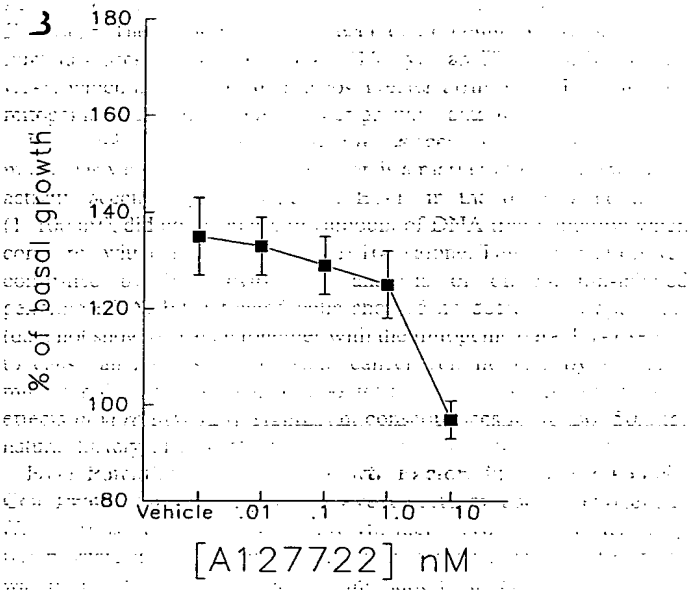
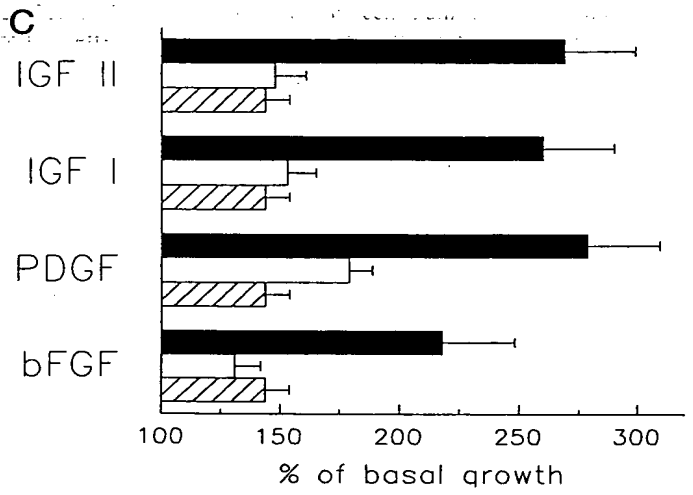


Fig. 1. Representative examples of ET-1 expression in human prostate cancer. *A*, primary prostate cancer, with specific ET-1 staining of the neoplastic cells. *B*, loss of ET-1 staining in a section flanking *A*, following immunoabsorption of the anti-ET-1 monoclonal antibody with ET-1. *C–F*, tissue obtained at autopsy from men dying of androgen-refractory prostate cancer. *C*, intense, specific ET-1 immunostaining of the neoplastic cells in primary prostate cancer; *D*, prostate cancer metastatic to lymph node, *E*, bone, with a typical osteoblastic response, and *F*, lung. *A–F*, × 40. Similar ET-1 staining was observed in every prostate cancer studied in 15 different men.







cells were a consistent positive internal control. These findings are consistent with the elevated levels of plasma-immunoreactive ET found in the majority of men with advanced metastatic disease and support our hypothesis that prostate cancer cell secretion may cause this increase (2). Interestingly, despite a decreased optimum antibody dilution (primary prostate cancer, 1:500; autopsy lesions, 1:1000) ET-1 immunostaining was generally more intense in the autopsy specimens; considering antibody dilution and staining intensity as rough indicators of peptide concentrations, levels of ET-1 are evidently higher in advanced prostate cancer specimens than in primary lesions.

ET-1-induced Prostate Cancer Cell Proliferation. In 4-day, serum-free growth assays, exogenous ET-1 induced proliferation in every established human prostate cancer cell line tested; the ratio of cell number to control (no ET-1 added) was significant (range, 10–50%) in each concentration (10 pM–100 nM) of ET-1 tested (data not shown). These data are in agreement with our previous observations conducted in the presence of insulin in the basal media (2), which can act as both a mitogenic factor and an inducer of ET-1 secretion (17). The ET-1-induced prostate cancer cell proliferation was blocked by the addition of the selective ET_A antagonist A-127722 (Fig. 2, *A* and *B*) but not by the selective ET_B antagonist BQ-788. These data support the hypothesis that the effects of ET-1 are mediated through ET_A only. The predominant ET receptor on benign prostatic epithelium has been identified as ET_B (16), yet an ET_B-specific ligand, ET-3, which has a more than 3-log greater affinity for ET_B, had no mitogenic effect on prostate cancer growth (data not shown).

ET-1 Does Not Decrease Prostate Cancer Cell Apoptosis. Low-molecular-weight DNA fragmentation is a marker of the endonuclease activity accompanying apoptosis. ET-1, in the dose range tested (1–100 nM), did not decrease the amount of DNA fragmentation when compared with cells grown in RPMI 1640 alone. This observation was confirmed by flow cytometric analysis of digoxigenin-labeled genomic DNA; ET-1-treated cells showed no decrease in apoptosis (data not shown). Taken together with the mitogenic data, ET-1 seems to cause an increase of prostate cancer cell number by inducing mitosis rather than decreasing apoptotic death; these modest growth effects *in vitro* may have significant consequences in the 30–50-year natural history of prostate cancer.

ET-1 Potentiates Multiple Growth Factors in Prostate Cancer Cell Proliferation. The proliferative effects of adding exogenous ET-1 (10 nM) to a polypeptide growth factor (10 ng) were tested in five prostate cancer cell lines. Synergistic prostate cancer cell growth was observed in 7 of 25 tested combinations, as demonstrated by a significant ($P < 0.05$) increase in cell number greater than the additive effects of ET-1 and the growth factor alone (Fig. 2*C*). Additive proliferative effects were observed in another 9 of 25 combinations, including EGF (LNCaP, PPC-1, and TSU), basic fibroblast growth factor (LNCaP), IGF-I (PC3 and PPC-1), IGF-II (PC3 and PPC-1), and PDGF (PC3). No synergistic growth effects were observed with EGF. The most significant increases were observed in the human prostate cancer cell line TSU and with the IGF-ET-1 combi-

Fig. 2. *A*, ET-1-stimulated proliferation of PPC-1 cells grown in RPMI 1640 (vehicle; ○) is inhibited by A-127722 (10 nM; ●), a selective ET_A antagonist. *B*, proliferation of PPC-1 cells grown in 1 nM ET-1 (■) is dose-dependently inhibited by A-127722 in the media. These data are representative of every prostate cancer cell line tested. *Point*, mean of quadruplicate determinations. *C*, synergistic growth effects of ET-1 (10 nM) added to a polypeptide growth factor (10 ng) (*solid bar*) compared with growth factor alone (*open bar*) and ET-1 alone (*hatched bar*) in the prostate cancer cell line TSU. The combinations (*solid bar*) represent a significant increase ($P < 0.05$, *t* test) over the additive effects of ET-1 and growth factor alone. *Bar*, mean of three experiments done in quadruplicate (±SE) as a percentage of basal growth (RPMI 1640 alone). Synergy was also observed between ET-1 and IGF-I, IGF-II, and PDGF in the human prostate cancer cell line DU145 (data not shown).

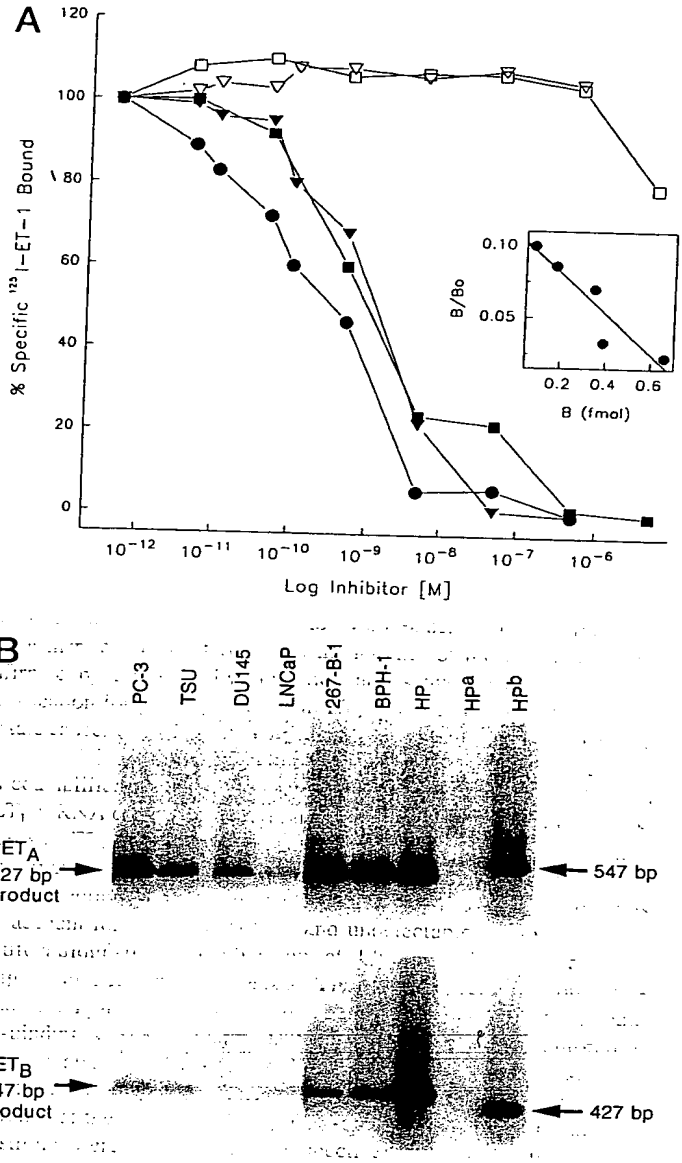


Fig. 3. *A*, representative example of the concentration-dependent inhibition of ^{125}I-labeled ET-1 binding to a prostate cancer cell line (PC-3) by ET-1 (●) and by the ET_A-selective ligands BQ-123 (▼) and A-127722 (■), but not by the ET_B-selective ligands sarafotoxin S6C (▽) or BQ-788 (□). Inhibition of ^{125}I-labeled ET-1 binding observed at the highest concentration of BQ-788 is presumably through antagonism at ET_A sites. Binding is expressed as a percentage of ^{125}I-labeled ET-1 bound in the absence of unlabeled ligand. *Point*, mean of triplicate determinations. *Inset*, Scatchard plot analysis of ^{125}I-labeled ET-1 saturation binding data to PC3. *B*, ET_A and ET_B mRNA detected by RT-PCR in human prostate cancer cell lines PC-3, TSU, DU145, and LNCaP, immortalized normal neonatal prostatic epithelial (267-B-1) and benign prostatic hyperplasia epithelial (BPH-1) lines, and human placenta (*HP*). The product of glyceraldehyde-3-phosphate dehydrogenase primer amplification (HP^a) fails to hybridize with either the ET_A or ET_B probes. The ET_A blot (*top*) was first hybridized with the ET_B probe, which revealed a band corresponding to the ET_B amplification product (HP^b); conversely, the ET_B blot (*bottom*) was hybridized first with the ET_A probe, which revealed a band corresponding to the ET_A amplification product (HP^b). No other bands were seen. After probe specificity was assured, the blots were hybridized with the appropriate probe, revealing the 427- and 547-bp ET_A and ET_B products, respectively.

nations. The growth-stimulatory effects of IGF-I have been demonstrated in primary prostate epithelial cells and may activate the androgen receptor and androgen signaling pathway directly (6). We have shown previously that ET-1 alone is a prostate cancer mitogen (2); as a facilitator of the more permissive growth factors, ET-1 may provide another mechanism of androgen-independent prostate cancer progression.

ET Receptors: Binding Sites and mRNA in Prostate Cancer Cell Lines. The ET receptor saturation studies performed *in situ* demonstrated that the binding of ^{125}I-labeled ET-1 was saturable and of high affinity in four of five human prostate cancer cell lines. Under the experimental conditions described, very little specific binding could be demonstrated in the prostate cancer cell line LNCaP; therefore, no definitive conclusions could be made about its K_d or B_{max}. In the other cell lines, the approximate K_ds and B_{max}s ranged from 0.08 (TSU) to 0.62 (PPC-1) nM and from 500 (PC3) to 23,700 (PPC-1) binding sites/cell, respectively, and are consistent with those reported for prostate and a number of other tissues (11–13; reviewed in Ref. 18). Competition binding experiments using ET_A-selective (BQ-123 and A-127722) and ET_B-selective (sarafotoxin S6C and BQ-788) receptor ligands demonstrated specific ET_A binding only (Fig. 3*A*). No ET_B binding was demonstrated in any of the human prostate cancer cell lines tested.

RT-PCR of total mRNA from human prostate cancer cell lines, immortalized normal human neonatal prostatic epithelial and benign prostatic hyperplasia epithelial lines, and human placenta (positive controls for ET_A and ET_B; Ref. 19) revealed a PCR product for both ET_A and ET_B after amplification for 35 cycles (Fig. 3*B*). The expected 427- and 547-bp PCR products were observed for the ET_A and ET_B primer sets, respectively, and the identity of these products was confirmed by Southern hybridization and sequence analysis. After amplification for 25 cycles, only an ET_A product was detected in the prostate cancer cell lines (data not shown). To assure the quality of the cDNA for PCR, human glyceraldehyde-3-phosphate dehydrogenase was coamplified in each case (data not shown). Although low levels of ET_B mRNA can be detected in the human prostate cancer cell lines, functional ET_B binding sites could not. It has been suggested that ligand-induced ET_B mRNA down-regulation may be due to increased message degradation (3); similar regulation in prostate cancer cells may account for low ET_B mRNA and undetectable protein.

Autoradiographic Localization of ET_A and ET_B Binding in Benign and Malignant Prostate Tissues. The reported predominance of ET_B-binding sites on the benign prostate epithelium and ET_A-binding sites in prostatic stroma was confirmed by autoradiographic analysis of ^{125}I-labeled ET-1 binding to prostate sections taken from five radical prostatectomy specimens (Fig. 4 and Table 1). The ratios of the densities of ET_A:ET_B-binding sites in the epithelium and stroma were 0.25 and 2.56, respectively, which are remarkably close to the ratios reported previously (0.20 and 2.54, respectively; Ref. 16). The infiltrating growth pattern of prostate cancer into an ET receptor-rich stromal background exceeded the autoradiographic resolution necessary to determine ET-binding sites on only malignant cells in the primary prostate cancer tissue sections examined. Despite this limitation, areas of cancer were quantified by pixel density and show decreased ET_B binding relative to other tissues. Certain prostate cancer metastases, such as the liver metastases obtained at autopsy, are characterized by homogenous sheets of malignant cells, and background ^{125}I-labeled ET-1 binding is not an issue. Clearly, ET_B binding was decreased greatly in these lesions. The liver tissue itself had roughly equal ET_A and ET_B binding (2.15 nCi/mg), as reported previously (20).

The specific functional consequences of the reduced ET_B expression in the human prostate cancer cell lines are unknown but provocative. The decreased expression or complete loss of ET_B receptor-specific responses could impact the secretion, function, and clearance of ET-1 in prostate cancer. For example, circulating ET-1 is cleared by ET_B receptors: ^{125}I-ET-1 binding *in vivo* is inhibited by BQ-788 (an ET_B antagonist), but not BQ-123 (an ET_A antagonist; Ref. 8). Furthermore, an i.v. infusion of this ET_B antagonist produced a significant increase in plasma ET-1 concentrations, suggesting that

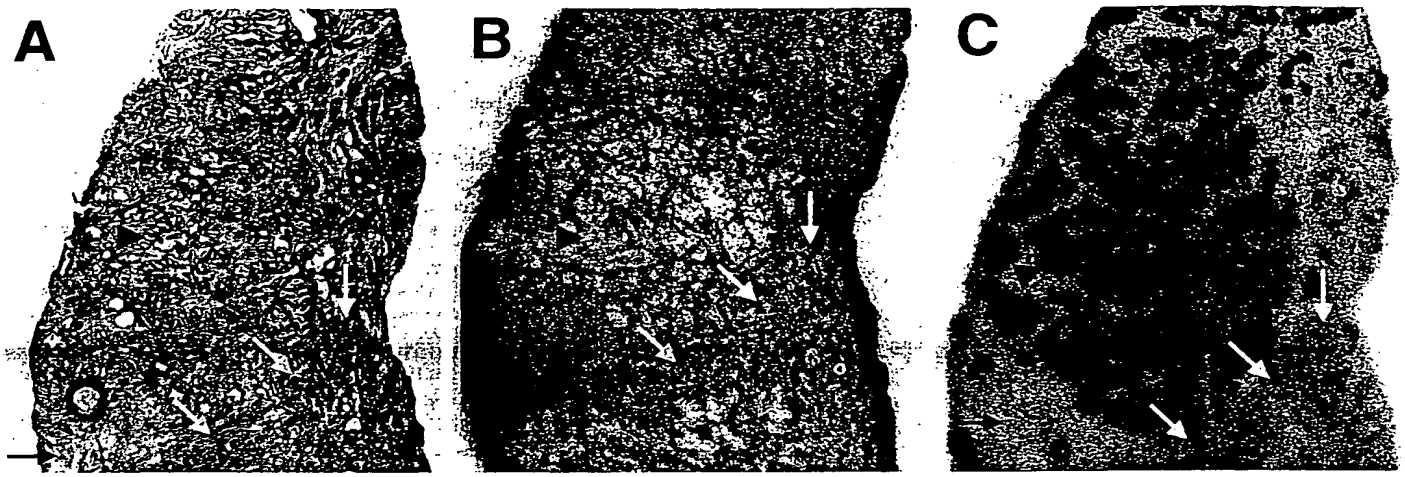


Fig. 4. ET-binding sites localized by ^{125}I-labeled ET-1 bound to human prostate tissue. *A*, human prostate tissue section stained with hematoxylin and eosin. *A–C, black arrow*, area of stroma; *black arrowhead*, area of benign prostate epithelium; *white arrow*, area of primary prostate cancer. *B*, total ET_A binding: autoradiogram of a flanking tissue section incubated with 0.1 nM ^{125}I-labeled ET-1 and 1 μM BQ-788 showing radioactive ligand binding primarily to the stromal elements. *C*, Total ET_B binding: autoradiogram of another flanking section incubated with 0.1 nM ^{125}I-labeled ET-1 and 1 μM BQ-123 showing radioactive ligand binding primarily to epithelial elements. The area of prostate cancer shows less ET_B binding than that of the benign epithelium. The addition of 1 μM unlabeled ET-1 reduced the binding to <25% total binding (not shown). × 8.

Table 1 *Specific ^{125}I-labeled ET-1 binding to prostate tissue by autoradiography*

Tissue	Radioactive density (nCi/mg)	
	ET_A binding[a]	ET_B binding[a]
Benign prostate epithelium	0.90 ± 0.13	3.57 ± 0.23[b]
Prostate stroma	5.53 ± 0.4[c]	2.16 ± 0.19[d,e]
Area of primary prostate cancer	2.15 ± 0.63	2.21 ± 0.74
Prostate cancer metastatic to liver	0.68 ± 0.12	0.84 ± 0.19

[a] Mean ± SE.
[b] $P < 0.0001$, ET_A *versus* ET_B, benign prostate epithelium, by Student's *t* test.
[c] $P < 0.0001$, ET_A, benign prostate epithelium *versus* prostate stroma.
[d] $P < 0.0001$, ET_A *versus* ET_B, prostate stroma.
[e] $P < 0.001$, ET_B, benign prostate epithelium *versus* prostate stroma.

ET_B blockade interrupts ET-1 clearance (8). A similar "hormonal buffer system" has been proposed for silent atrial natriuretic receptors (21). Finally, ET-1 secretion from cultured human keratinocytes, which express ET_B exclusively, is inhibited by exogenous ET-1 (22). These observations may explain, in part, the increased tissue and plasma concentrations of ET-1 seen in advanced prostate cancer.

The loss of another ET_B-specific response may impact prostate cancer progression. The vasodilatory effect of the ET isopeptides has been linked to ET_B-mediated NO production counteracting otherwise unopposed ET_A-induced vasoconstriction (23, 24). NO has been shown to counter-regulate ET-1 broadly, by blunting both receptor binding and subsequent Ca^{2+} elevation and by inhibiting ET-1 production by the endothelium (25, 26). Although its exact role in tumor biology is still unclear, NO induces cytotoxicity and apoptosis *in vitro* and has been shown to both inhibit metastases and, conversely, to promote tumor growth *in vivo* (27–30). If the NO present in prostate glandular epithelium (31) is under similar ET_B-mediated regulation, then the loss of this receptor in prostate cancer may impact tumor progression directly.

Disruption of the *ET_B* gene, or its selective ligand, ET-3, produces homozygous mice with a regional lack of neural crest-derived enteric neurons and melanocytes (32, 33). These phenotypic changes (aganglionitic megacolon and coloration) are similar to the Piebald-Lethal mouse and Hirschsprung's disease in humans. A missense mutation of the *ET_B* gene has been identified in multigenic Hirschsprung's disease (34) and maps to human chromosome *13q22* (35). This region is a frequent target of allelic loss in primary prostate cancer, suggesting an inactivation event in pathogenesis. Whether the *ET_B* locus, as a negative regulator of ET-1 and ET_A, functions like a prostate cancer tumor suppressor gene is actively under investigation.

These data indicate clearly that the lethal form of advanced prostate cancer has an ET-1-secreting phenotype at metastatic sites as well as the primary tumor. As a mitogen and potentiator of other growth factors for prostate cancer proliferation, ET-1, acting through ET_A, provides a distinct therapeutic target.

Acknowledgments

We thank Michele Bartkowski, Pearl Campbell, Jonathan Walsh, Stuart Criley, Gordon Wiegand, Nozomu Inoue, and Ronald Rodriguez for technical assistance.

References

1. Wingo, P. A., Tong, T., and Bolden, S. Cancer statistics, 1995. CA Cancer J. Clin. *45:* 8–30, 1995.
2. Nelson, J. B., Hedican, S. P., George, D. J., Reddi, A. H., Piantadosi, S., Eisenberger, M. A., and Simons, J. W. Identification of endothelin-1 in the pathophysiology of metastatic adenocarcinoma of the prostate. Nat. Med., *1:* 944–949, 1995.
3. Rubanyi, G. M., and Polokoff, M. A. Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. Pharmacol. Rev., *46:* 325–415, 1994.
4. Battistini, B., Chailler, P., D'Orleans-Juste, P., Briere, N., and Siros, P. Growth regulatory properties of endothelins. Peptides, *14:* 385–399, 1993.
5. Steiner, M. S. Role of peptide growth factors in prostate: a review. Urology, *42:* 99–110, 1993.
6. Culig, Z., Hobisch, A., Cronauer, M. V., Radmayr, C., Trapman, J., Hittmair, A., Bartsch, G., and Klocker, H. Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. Cancer Res., *54:* 5474–5478, 1994.
7. Opgenorth, T. J., Adler, A. L., Calzadilla, S., Chiou, W. J., Dayton, B. D., Dixon, D. B., Gehrke, L. J., Hernandez, L., Magnuson, S. R., Marsh, K. C. Novosad, E. I., von Geldern, T. W., Wessale, J. L., Winn, M., and Wu-Wong, J. R. Pharmacological characterization of A-127722: an orally active and highly potent ET_A-selective receptor antagonist. J. Pharmacol. Exp. Ther., in press, 1995.
8. Fukahiro, T., Fujikawa, T., Ozaki, S., Ishikawa, K., Yano, M., and Nishikibe, M. Clearance of circulating endothelin-1 by ET_B receptors in rats. Biochem. Biophys. Res. Commun., *199:* 1461–1465, 1994.
9. Wyllie, A. H. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature (Lond.), *284:* 555–556, 1980.
10. Carducci, M. A., Nelson, J. B., Chan-Tack, K. M., Ayyagari, S., Sweatt, W. S., Campbell, P. S., Nelson, W. G., and Simons, J. W. Phenylbutyrate induces apoptosis in human prostate cancer and is more potent than phenylacetate. Clin. Cancer Res., *2:* 379–387, 1995.
11. Kobayashi, S., Tang, R., Wang, B., Opgenorth, T., Langenstroer, P., Shapiro, E., and Lepor, H. Binding and functional properties of endothelin receptor subtypes in the human prostate. Mol. Pharmacol., *45:* 306–331, 1994.
12. MacCumber, M. W., Ross, C. A., and Synder, S. H. Endothelin in brain: receptors, mitogenesis, and biosynthesis in glial cells. Proc. Natl. Acad. Sci. USA, *87:* 2359–2363, 1990.

13. Kusuhara, M., Yamaguchi, K., Kuranami, M., Suzaki, A., Ishikawa, S., Moon, H., Adachi, I., Hori, S., and Handa, S. Stimulation of anchorage-independent cell growth by endothelin in NRK 49F cells. Cancer Res., *52:* 3011–3014, 1992.
14. Cyr, C., Huebner, K., Druck, T., and Kris, R. Cloning and chromosomal localization of a human endothelin ET_A receptor. Biochem. Biophys. Res. Commun., *181:* 184–190, 1991.
15. Nakamuta, M., Takayanagi, R., Sakai, Y., Sakamoto, S., Hagiwara, H., Mizuno, T., Saito, Y., Hirose, S., Yamamoto, M., and Nawata, N. Cloning and sequence analysis of a cDNA encoding human non-selective type of endothelin receptor. Biochem. Biophys. Res. Commun., *177:* 34–39, 1991.
16. Kobayashi, S., Tang, R., Wang, B., Opgenorth, T., Stein, E., Shapiro, E., and Lepor, H. Localization of endothelin receptors in the human prostate. J. Urol., *151:* 763–766, 1994.
17. Oliver, F. J., de la Rubia, G., Feener, E. P., Lee, M-E., Loeken, M. R., Shiba, T., Quertermous, T., and King, G. L. Stimulation of endothelin-1 gene expression by insulin in endothelial cells. J. Biol. Chem., *266:* 23251–23256, 1991.
18. Huggins, S. P., Pelton, J. T., and Miller, R. C. The structure and specificity of endothelin receptors: their importance in physiology and medicine. Pharmacol. & Ther., *59:* 55–123, 1993.
19. Elshourbagy, N. A., Korman, D. R., Wu, L. H., Sylvester, D. R., Lee, J. A., Nuthalaganti, P., Bergsma, D. J., Kumar, C. S., and Nambi, P. Molecular characterization and regulation of the human endothelin receptors. J. Biol. Chem., *268:* 3873–3879, 1993.
20. Jouneaux, C., Mallat, A., Serradeil-Le Gal, C., Goldsmith, P., Hanoune, J., and Lotersztajn, S. Coupling of endothelin B receptor to the calcium pump and phospholipase C via Gs and Gq in rat liver. J. Biol. Chem., *269:* 1845–1851, 1994.
21. Maack, T., Suzuki, M., Almeida, F. A., Nussenzveig, D., Scarborough, R. M., McEnroe, G. A., and Lewicki, J. A. Physiological role of silent receptors of atrial natriuretic factor. Science (Washington DC), *238:* 675–678, 1987.
22. Yohn, J. J., Smith, C., Stevens, T., Morelli, J. G., Shurnas, L. R., Walchak, S. J., Hoffman, T. A., Kelley, K. K., Escobedo-Morse, A., Yanagisawa, M., Kane, M. A., and Zamora, M. R. Autoregulation of endothelin-1 secretion by cultured human keratinocytes via the endothelin B receptor. Biochim. Biophys. Acta, *1224:* 454–458, 1994.
23. Warner, T. D., de Nucci, G., and Vane, J. R. Rat endothelin is a vasodilator in the isolated perfused mesentery of the rat. Eur. J. Pharmacol., *159:* 325–326, 1989.
24. Hirata, Y., Emori, T., Eguchi, S., Kanno, K., Imai, T., Ohta, K., and Marumo, F. Endothelin receptor subtype B mediates synthesis of nitric oxide by cultured bovine endothelial cells. J. Clin. Invest., *91:* 1367–1373, 1993.
25. Goligorski, M. S., Tsukahara, H., Magazine, H., Andersen, T. T., Malik, A. B., and Bahou, W. F. Termination of endothelin signaling: role of nitric oxide. J. Cell. Physiol., *158:* 485–494, 1994.
26. Boulanger, C., and Lüscher, T. F. Release of endothelin from the porcine aorta: inhibition by endothelin-derived nitric oxide. J. Clin. Invest., *85:* 587–590, 1990.
27. Kitasjima, I., Kawahara, K., Nakajima, T., Soejima, Y., Matsuyama, T., and Maruyama, I. Nitric oxide-mediated apoptosis in murine mastocytoma. Biochem. Biophys. Res. Commun., *204:* 244–251, 1994.
28. Corasaniti, M. T., Melino, G., Tartaglia, R. L., Finazzi-Agro, A., and Nistico, G. *N*-methyl-D-aspartate-induced excessive formation of nitric oxide in CHP100 neuroblastoma cells produces death of BMEL melanoma cells in co-culture. Neuropharmacology, *33:* 1071–1077, 1994.
29. Xie, K., Huang, S., Dong, Z., Juang, S-H., Gutman, M., Xie, Q-W, Nathan, C., and Fidler, I. J. Transfection with the inducible nitric oxide synthase gene suppresses tumorigenicity and abrogates metastasis by K-1735 murine melanoma cells. J. Exp. Med., *181:* 1333–1343, 1995.
30. Jenkins, D. C., Charles, I. G., Thomsen, L. L., Moss, D. W., Holmes, L. S., Baylis, S. A., Rhodes, P., Westmore, K., Emson, P. C., and Moncada, S. Roles of nitric oxide in tumor growth. Proc. Natl. Acad. Sci. USA, *92:* 4392–4396, 1995.
31. Burnett, A. L. Nitric oxide control of lower genitourinary tract functions: a review. Urology, *45:* 1071–1083, 1995.
32. Hosoda, K., Hammer, R. E., Richardson, J. A., Greenstein-Baynash, A., Cheung, J. C., Giaid, A., and Yanagisawa, M. Targeted and natural (piebald-lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice. Cell, *79:* 1267–1276, 1994.
33. Greenstein-Baynash, A., Hosoda, K., Giaird, A., Richardson, J. A., Emoto, N., Hammer, R. E., and Yanagisawa, M. Interaction of endothelin-3 with endothelin-B receptor is essential for development of epidermal melanocytes and enteric neurons. Cell, *79:* 1277–1285, 1994.
34. Puffenberger, E. G., Hosoda, K., Washington, S. S., Nakao, K., deWit, D., Yanagisawa, M., and Chakravarti, A. A missense mutation of the endothelin-B receptor gene in multigenic Hirschsprung's disease. Cell, *79:* 1257–1266, 1994.
35. Puffenberger, E. G., Kauffman, E. R., Bolk, S., Matise, T. C., Washington, S. S., Angrist, M., Weissenbach, J., Garver, K. L., Mascari, M., Ladda, R., Slaugenhaupt, S. A., and Chakravarti, A. Identity-by-decent and association mapping of a recessive gene for Hirschsprung's disease on human chromosome 13q22. Hum. Mol. Genet., *3:* 1217–1225, 1994.